Rapid Increase in Serum Iron Level After Oral Iron Intake as an Indicator of Duodenal Iron Absorption and Inverse Regulation of Iron Absorption by Hepcidin Expression

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Abstract: **Objective:** Hepcidin is a hepatocyte-derived peptide that is thought to be involved in the regulation of intestinal iron absorption. Ferric citrate is a phosphate binder with additional effects on iron absorption. Better understanding of iron absorption under various levels of hepcidin may improve ferric citrate supplementation in individuals with renal anemia. We provide a new method to predict the individual iron absorption ability of the duodenum. **Methods:** Rats on an ordinary diet were given 10 mg of ferric citrate, and the serum concentrations of hepcidin and iron were monitored for 24 hours. Rats with hepcidin levels induced by using alternative methods such as bloodletting or intravenous iron loading were also given ferric citrate, and serum iron level was measured at 2 hours after oral iron intake (2-hour oral iron absorption test). **Results:** Serum iron level increased constantly within 2 hours after oral iron intake, and serum hepcidin level peaked 4 hours after the iron level peaked. In the oral iron absorption test, the hepcidin levels inversely correlated with increased serum iron levels, and hepcidin expression levels of >80 ng/ml completely inhibited the increase in iron absorption. **Conclusion:** This study suggests that hepcidin expression may be a strong mediator to regulate iron absorption and that performing an oral iron absorption test with hepcidin may help improve oral iron dosing schedules in patients undergoing hemodialysis.

**Key Words:** hepcidin, ferroportin, divalent metal transporter 1, iron absorption, ferric citrate

Almost all patients on maintenance hemodialysis have iron deficiency anemia because of blood loss. The amount of iron loss has been estimated to be 1.5 to 2.0 g/year (1). Thus, adequate iron supplementation, typically requiring non-physiological intravenous iron administration, has become routine for hemodialysis patients (2), because oral iron supplementation has been shown to be unable to maintain adequate iron stores (3-5). However, the adequate iron stores in patients and the efficacy of the physiological iron absorption route in the intestine are controversial.

The physiological properties of iron metabolism in humans are characterized by the absence of active excretory mechanisms for iron and the regulation of body iron content by modulation of iron absorption in the duodenum (6). Iron is absorbed by an active transport mechanism comprised of two steps, namely mucosal uptake of iron from the lumen and mucosal transfer of iron to the bloodstream (7). Both steps are regulated by the body’s demand for iron. The phenomenon is supported by the transporter molecules divalent metal transporter (DMT-1) and ferroportin (FPN) (8). Iron influx from the intestinal lumen to cells is induced by DMT-1 expression, and iron efflux from cells to the bloodstream is induced by FPN expression. These unique mechanisms for cellular iron uptake and transport have evolved to prevent the inherent toxicity of iron in the promotion of oxidation due to iron overloading (9). However, many broad variations in iron status have been observed among individuals, and accurate

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regulations according to physiological demand remain unknown. Assays for DMT-1 and FPN expression in individual patients are not available in clinical settings.

Hepcidin is the main hormone/peptide that regulates plasma iron levels by controlling the iron export function of FPN, thereby regulating iron absorption and distribution (10). Measurement of hepcidin expression level is progressively being validated (11-13). Currently, its clinical value needs to be assessed in different physiological situations. Based on these molecular properties, we aimed to clarify the iron absorption in individual rat models. In previous ferrokinetic studies, iron stable isotopes were used in the analyses of iron absorption in the intestine (7, 14-16). However, in the clinical field, isotopes are of no practical use; thus, an alternative method that does not use isotopes is expected to detect iron absorption in individual cases.

The aims of this study were to quantify the magnitude of iron absorption under different hepcidin levels and to develop a new method to predict the bioavailability of oral iron in individual cases without isotope-labeled iron. In this study, we focused on basic serum hepcidin levels and the changes in serum iron concentration 2 hours after iron intake.

Materials and methods

1. Animals

Rats (male Sprague-Dawley rats aged 7 to 8 weeks) were obtained from Sankyo Laboratory (Japan) and maintained on a standard rodent diet (iron content: 20 mg/100 g). Synthetic human hepcidin-25 was purchased from Peptide Institute (Japan) and dissolved to appropriate concentrations in phosphate-buffered saline (PBS). Rats received a single intravenous injection of 100 μg of human hepcidin-25. Sucrose iron (40 mg/2 ml; Fesin, Nichi-Iko Pharmaceutical Co., Japan) was used for the injection. Sucrose iron was diluted in 4 mg/ml PBS. Rats received a 4 to 12 mg of iron through intravenous injections of 0.5 ml of iron solution 2 to 6 times a week. A relatively mild protocol of iron overload was chosen to evaluate the relationship between iron absorption and hepcidin expression level under physiological conditions. The control rats received PBS only at the same time points. For inhibiting endogenous hepcidin expression, iron deficiency rats were induced by 1 ml of bloodletting 2 to 6 times a week. For the iron absorption study, the rats were given 10 mg of ferric citrate (FC), including 2.3 mg of iron (Wako, Japan) in 0.5 ml of PBS, by oral gavage at 8:00 am. All the experiments were approved by the animal research committee of Kanazawa Medical University.

2. Blood parameter measurement

The rats were anesthetized by using 30% (v/v) isoflurane and killed by exsanguination. Whole blood samples were collected by abdominal aortic puncture for measurements of serum iron level, unsaturated iron-binding capacity (UIBC) and serum hepcidin level. Serum iron level and UIBC were measured by using 2-nitroso-5-(N-sulfo)propylamino) phenolhydrochloride and ascorbic acid. Serum hepcidin level was measured by using liquid chromatography coupled with tandem mass spectroscopy with reversed phase extraction (Medical Care Proteomics Biotechnology Ltd., Japan) (11).

3. Statistical analysis

Statistical analyses were performed by using Statistical package for Social Science (SPSS) Statistics 22 (IBM, USA). The Student t test was used to compare groups of parametric data. Simple linear regression analysis was used to examine the relationship between serum hepcidin levels and changes in serum iron level 2 hours after iron intake. Variables that were not normally distributed were log transformed before the analysis. Data are presented herein as mean ± standard error of the mean. Statistical significance was defined when the p value was <0.05.

Results

1. Serum iron levels after oral iron intake

In a time-course study, the rats (n=45 rats) were administered 10 mg of FC by oral gavage at 8:00 am and killed at 0, 0.5, 1, 2, 3, 6, 9, 12, and 24 hours after FC intake (n = 5 rats in each group). The onset of the increase in serum iron level after FC intake was quite rapid (Fig 1). Serum iron and transferrin saturation levels (TSAT) increased within 30 minutes and reached the maximum value (488 ± 57.6 μg/dl, 95 ± 2.1%) 2 hours after iron intake (Fig 1A,B). In the rats on a normal diet, the mean pretreatment serum iron concentration was 236 ± 39.5 μg/dl. Thereafter, the serum iron level decreased to its baseline value. On the other hand, the serum hepcidin level gradually increased to its maximum value (56.9 ± 10.5 ng/ml) 4 hours after the serum iron level peaked (Fig 1C).

To clarify whether the rats maintained the circadian rhythm of serum iron, their serum iron levels were measured at 8:00, 14:00, and 20:00 (n = 5 rats in each group). The serum iron levels were high in the morning and low in the evening (Fig 2). Under consideration of the circadian rhythm in the rats, all of the rats received FC at 8:00 am.

Based on these data, the change in serum iron level at 2 hours after oral iron intake was substituted for iron absorption, which was named as 2-hour oral iron absorption test (2-h OIAT).
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2. Effects of exogenous hepcidin expression on 2-h OIAT results

To study the acute effect of exogenous hepcidin expression, the rats were injected intravenously with 0-, 25-, 50-, and 100-μg doses of human hepcidin-25 at 7:00 am, given 10 mg of FC by oral gavage at 8:00 am, and then killed at 10:00 am (n = 5 rats in each group). Increasing the hepcidin-25 dose resulted in a progressive decrease in serum iron concentration (Fig 3A). In the 2-h OIAT, the rats given 100 μg of hepcidin-25 showed significantly lower serum iron levels than the control rats (268 ± 47 μg/dl vs 501 ± 28 μg/dl, p<0.05). Serum human hepcidin-25 concentration was dependent on the amount of hepcidin-25 injected (Fig 3B).

3. Effects of endogenous hepcidin expression on 2-h OIAT results

To suppress endogenous hepcidin expression, 10 rats were bled 1 ml of whole blood per day, 5 times a week (iron deficiency group). To stimulate endogenous hepcidin expression, 20 rats were given 4 or 12 mg of iron through 2 mg of iron injection per day, 2 or 6 times a week (mild and severe iron loading groups, n = 10.
rats in each group). The controls had saline only (10 rats). After 2 days of treatment holiday, the 40 rats were divided into 2-h OIAT and control groups, received 10 mg of FC or saline by oral gavage at 8:00 am, and then killed at 10:00 am. As expected, the hepcidin levels were significantly reduced in the iron deficiency group (2.2 ± 1.5 ng/ml, p<0.05) and significantly increased in the iron loading groups (67.5 ± 6 ng/ml in the mild group and 120 ± 24 in the severe group, p<0.05, respectively) when compared with the control group (26.3 ± 5.3 ng/ml; Figure 4B). Baseline serum iron levels were significantly increased in the iron deficiency group when compared with the severe iron loading group (Figure 4A). The serum iron level in the 2-h OIAT was significantly reduced in the iron loading groups when compared with the control group (245.7 ± 22.9 in the mild group, 211.8 ± 17.3 in the severe group, p<0.05, p<0.05, respectively, and 488.0 ± 57.6 ng/ml in the control group). There was no significant difference in serum iron levels between the iron deficiency and control groups.

4. Effect of endogenous hepcidin expression after the first oral iron intake on the second iron intake

The effect of serial iron intake on iron absorption was examined because increase in hepcidin level peaked at 6 hours after the first oral intake (Fig 1). The rats received a second 10-mg dose of ferric citrate at 2, 3, or 6 hours after first iron intake (n = 5 rats in each group). The serum iron levels in the 2-h OIAT were significantly reduced at 6 hours when compared with the control at 8:00 am (384 ± 33 μg/dl vs 488±57.6 μg/dl, p<0.05; Fig 5).

5. Changes in serum iron level induced by 2-h OIAT in the individual rats

Next, the effects of endogenous hepcidin expression on iron absorption in iron deficiency, control and iron loading groups (Fig 5) were confirmed by using 2-h OIAT in the individual rats, which had various hepcidin levels. To induce various hepcidin levels, 14 rats bloodletting (2 ml in 3 rats and...
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Discussion

The results of our study show that increased serum iron level 2 hours after iron intake is a useful alternative to ferrokinetic analysis using radiolabeled iron for evaluating iron absorption. In addition, our study shows that the increased serum iron levels inversely correlated with basic serum hepcidin levels.

The serum iron levels of the rats reflects the circadian rhythm, and the iron levels were high in the early morning and low in the late evening, similarly to those described in previous reports (17, 18). Accordingly, the rats were administered FC at 8:00 am in our study. Although we did not use a radioisotope as a ferrokinetic study, the serum iron concentration was rapidly increased within 30 minutes and peaked constantly at 2 hours after iron intake apart from the circadian rhythm. Furthermore, the onset of iron absorption in the duodenum was extremely rapid. Wheby et al (7) reported that radiolabeled iron was detected in rat bodies as early as 15 seconds after the iron reached the closed loop of the duodenum and varied in peak levels at 1 hour. These findings implied that increased serum iron level may reflect iron absorption just after influx from the stomach to the duodenum and that the 2-hour iron absorption test may be useful for the evaluation of iron absorption in individual cases. High correlation was observed between the absorption of isotope-labeled iron and the change in serum iron level 6 hours after intake of 100 mg of iron in humans (19).

Rivera et al (20) reported that hepcidin accumulated in the FPN-rich organs, namely the liver, spleen, and duodenum. They also reported that the rapid action
of a single 50-µg dose of human hepcidin-25 administered to mice makes human hepcidin-25 an appealing agent for the prevention of iron release via FPN from iron-storing organs, which consequently shows hypoferric effect within 1 hour. Laftah et al (21) reported that administration of exogenous human hepcidin-25 significantly reduced the mucosal uptake and transfer to the body in mice given isotope-labeled iron. In our study, a single 100-µg dose of exogenous human hepcidin-25 inhibited the increase in serum iron in the 2-h OIAT, which might reflect iron absorption. Although we did not measure the DMT-1 expression, hepcidin may reduce iron absorption by both inhibiting enterocyte DMT-1 transcription and binding to FPN on the enterocyte basolateral membrane (22, 23).

Correlation has been reported between endogenous serum hepcidin level and iron absorption (15, 16, 24, 25). Cao et al (15) reported that radiolabeled ferrokinetic studies in rats revealed that liver hepcidin mRNA expression inversely correlated with nonheme iron absorption, although their trials were far from clinical application in individual cases. Others (16, 24, 25) reported that plasma hepcidin is a modest predictor of dietary iron bioavailability in healthy men and women with iron statuses ranging from iron deficiency to iron sufficiency. Eschbach et al (14) reported the quantitative relationship between serum level of ferritin, which is a regulator of hepcidin expression via bone morphogenetic protein (BMP) 6 (26), and iron absorption in healthy subjects and patients undergoing long-term dialysis. In all the aforementioned previous studies, iron absorption was evaluated by measuring the amount of isotopic iron incorporated into red blood cells or in whole blood at 2 weeks after ingestion of isotope-labeled iron. The methods are accurate but difficult to implement in individual subjects. Although our method is simple and takes only 2 hours to perform, our findings about the correlation between baseline serum hepcidin levels and changes in serum iron levels in the 2-h OIAT were similar to the results of the previous studies. Our method is simple and provides a means for studying iron absorption without the use of isotope-labeled iron. Furthermore, based on the inverse correlation curve, we can infer the upper limit of hepcidin to inhibit iron absorption completely in patients undergoing hemodialysis. In this regard, much more studies are necessary in clinical fields.

In the present study, oral iron loading increased circulating hepcidin level, which in turn affected iron absorption, followed by a secondary iron intake. Transferrin receptor (TFR) 2 expression modulates the signal between iron status and hepcidin expression via the Bmp6/Smad pathway (27). This might take 4 hours to induce hepcidin expression from TFR2 activation by the alteration of serum iron level. Reduced efficacy of a second iron supplementation by the increased hepcidin level may act as a feedback mechanism to limit iron efflux into plasma. Furthermore, the amount of oral iron intake is a main factor of the inhibition of iron uptake via DMT-1 (28). DMT-1 expression is regulated by intracellular iron-regulatory proteins, which is regulated by the amount of intracellular iron. When oral iron is loaded, DMT-1 expression is reduced via the iron-regulatory protein pathway and then iron uptake is blocked. We did not perform additional measures after 9 hours and thus could not clarify whether further decrease in serum iron level occurred after the 2-h OIAT. Further studies are needed to analyze the mechanisms of iron absorption via hepcidin and DMT-1 expressions.

In the previous studies, oral iron supplementation was unable to maintain adequate iron stores and treat iron deficiency in patients undergoing hemodialysis (3-5). Subsequently, the use of intravenous iron injection in patients with end-stage renal disease has become widespread. Oral iron supplementation often causes gastric irritation, nausea, and constipation, which may decrease treatment adherence and long-term efficacy. High hepcidin levels induced by severe ferritinemia block iron absorption. FC was recently developed as a phosphate binder. Treatment with FC can induce increases in iron parameters while maintaining hemoglobin levels, with a safety profile and without decreased drug adherence. In this situation, prediction of the bioavailability of oral iron may be important in making oral iron dosing schedules for individual patients undergoing hemodialysis who have low iron absorption, probably because of excessive iron stores and high hepcidin levels induced by intravenous iron injection, low levels of erythropoiesis or both.

**Conclusion**

Our data suggest that hepcidin level may be the main regulating factor of iron absorption in the intestine. Based on the relationship between hepcidin and serum iron levels 2 hours after iron intake, a new method for ferrokinetics can be developed in order to analyze iron absorption without isotope and to speculate the reason for the lack of iron absorption. The application of the 2-h OIAT in clinical fields is expected in the future. At the start of hemodialysis, a blood sample is drawn for measurement of serum iron and hepcidin-25 levels before FC ingestion and again a small amount of blood is drawn for serum iron measurement 2 hours after oral iron intake.

The present study suggests that inducing high hepcidin levels (>80 ng/ml) minimizes fractional iron absorption. The 2-h OIAT may support alternate-day
supplementation. This analytical system can be applied in clinical fields in order to estimate the efficacy of oral iron administration in individual cases. It can also be established as a standard method for determining the upper-limit hepcidin level for inhibiting iron accumulation in different physiological and/or pathological situations.

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Conflict of interest

One author (N.T.) is the founder of Medical Care Proteomics Biotechnology Co., Ltd. The other authors declare that they have no competing interests.

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